



## Effects of Different Lipophilized Ferulate Esters in Fish Oil-Enriched Milk: Partitioning, Interaction, Protein, and Lipid Oxidation

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**Effects of different lipophilized ferulate esters in fish oil enriched milk: Partitioning, Interaction, Protein and Lipid Oxidation**

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***Effects of different lipophilized ferulate esters in fish oil enriched milk:  
Partitioning, Interaction, Protein and Lipid Oxidation***

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**ABSTRACT:** Antioxidant effects of ferulic acid and lipophilized ferulate esters were investigated in fish oil enriched milk. Methyl ferulate (C1) and ethyl ferulate (C2) more efficiently prevented lipid oxidation than dodecyl ferulate (C12), followed by ferulic acid (C0). The combination of C1 or C2 with C12 could have a “synergistic” effect indicated by peroxide value, hexanal, and 1-penten-3-ol analysis results. These antioxidants also showed protein oxidation inhibition effects. The most effective antioxidants (C1 and C2) had the highest concentration in the precipitate phase, but lowest concentration in aqueous phase which was opposite to the partitioning of C0. C12 had the highest concentration in the oil and emulsion phase. In particular, the interaction between ferulates esterified with short and medium alkyl chain lengths respectively could lead to their “synergistic” effects in fish oil enriched milk, which could be caused by the change of their partitioning or localization at the interface.

**KEY WORDS:** Ferulic acid, Ferulate ester, omega-3 PUFA, protein oxidation, lipid oxidation, antioxidants, antioxidant partitioning, phenolipids

## INTRODUCTION

Fish oils are reported to have many health beneficial effects including reduced risk of cardiovascular and inflammatory diseases. This is attributed to the presence of long chain n-3 polyunsaturated fatty acids (PUFAs).<sup>1,2</sup> It is thus of great interest to the food industry to enrich food with fish oil. However, fish oils that contain n-3 PUFA like eicosapentaenoic acid (EPA, 20:5) and docosahexanoic acid (DHA, 22:6) are highly prone to lipid oxidation. Lipid oxidation can cause development of compounds with an unpleasant off flavor in the product even at low concentrations (e.g. 1 µg/kg). Moreover, lipid oxidation may also generate some oxidized compounds, which are potentially harmful to human health.<sup>3-5</sup> Therefore, the use of efficient antioxidants in food is required to limit oxidation of the healthy n-3 PUFAs and other compounds in the food product.

Currently, there is an increasing interest in using natural antioxidants such as phenolic acids in foods. However, phenolic acids could have limited effectiveness in oil-in-water (O/W) emulsions since they are generally hydrophilic and have relatively high polarity. Polar antioxidants are less efficient in O/W emulsions than apolar antioxidants according to the “polar paradox” theory put forwarded first by Porter<sup>6</sup> and Porter et al.<sup>7</sup> This was explained by the partitioning of the antioxidants into the different phases. Apolar antioxidants were closely located to the interface, where lipid oxidation is initiated, whereas polar antioxidants are mainly located in the aqueous phase. The polarity/hydrophilicity of phenolic acids has therefore been modified by esterification with fatty alcohols in order to change the partitioning of the antioxidant in the O/W emulsion system to get better antioxidant effects.<sup>8</sup> Interestingly, the lipophilized phenolic acids have a higher antioxidant activity in O/W emulsions with the increment of alkyl chain length but only to a certain limit termed critical chain length. With further increment of the chain length, the antioxidant effect was significantly decreased. Based on these results, the cut-off effect was introduced.<sup>9</sup> The

mechanism of the cut-off phenomenon is still not very clear despite the fact that there has been significant progress in this area recently.<sup>10</sup>

In particular, there are only a few studies on antioxidant efficacies of lipophilized phenolic acids in real food systems. A study on antioxidant effects of lipophilized caffeic acid has shown that methyl and butyl caffeates were most efficient in milk, whereas butyl, octyl and dodecyl caffeates were most efficient in mayonnaise, suggesting that the critical chain lengths of lipophilized phenolic compounds were different in different foods enriched with fish oil.<sup>11</sup> Moreover, a study on dihydrocaffeates and rutin esters in fish oil enriched milk showed that phenolipids with medium chain were more efficient although phenolipids with only two different alkyl chain lengths were evaluated.<sup>12</sup> In another study with lipophilized ferulic acid, methyl ferulate had the best antioxidant activity of all the ferulates and ferulic acid evaluated in fish oil enriched milk. Octyl and dodecyl ferulates were the least efficient compounds evaluated in this study where they acted as prooxidants in spite of their more apolar character than methyl ferulate.<sup>13</sup>

It is known that a combination of antioxidants can often provide synergistic antioxidant efficiency although the mechanism is not completely understood. To the best of our knowledge, there have been no studies performed on a combination of antioxidants of lipophilized ferulic acids with different alkyl chain lengths. Therefore, it is of interest to test the antioxidant efficiency of the mixed lipophilized ferulic acids. Based on former experiment with fish oil enriched milk<sup>13</sup>, the combination of ferulate esters with short alkyl chain length and medium alkyl chain length was selected. In addition, only few studies have evaluated the partitioning of antioxidants in fish oil enriched milk as well as the effect of phenolipids on protein oxidation. Thus, the first aim of this study was to evaluate the effects of ferulic acid, ferulate esters, and a combination of short and medium alkyl chain ferulate esters on the oxidative stability of fish oil enriched milk. Both lipid and

protein oxidation was assessed. The second aim was to compare the oxidative stability to the partitioning of ferulic acid and ferulates in fish oil enriched milk.

## MATERIALS AND METHODS

**Materials.** Pasteurized milk with 0.4% and 1.5% fat content was purchased from a local supermarket. Cod liver fish oil was supplied by Maritex A/S (TINE BA, Sortland, Norway). The peroxide value (PV) of the fish oil was 0.65 meq peroxides/kg. The fish oil had 242 mg  $\alpha$ -tocopherol/kg, 119 mg  $\gamma$ -tocopherol/kg, 44 mg  $\delta$ -tocopherol/kg, respectively. The fatty acid composition of the oil was analyzed by GC as: C14:0, 3.8%; C16:0, 9.9%; C16:1 (n-7), 9.4%; C18:0, 2.0%; C18:1 (n-9), 17.2%; C18:1 (n-7), 4.8%; C18:2 (n-6), 2.0%; C18:3 (n-3), 2.8%; C18:4(n-3), 0.2%; C20:1, 13.2%; C20:5 (n-3) (EPA), 9.8%; C22:1(n-11), 5.8%; C22:5(n-3), 1.2%; C22:6 (n-3) (DHA), 11.9% (w/w).

Methyl (C1) and dodecyl (C12) ferulates used in the study were synthesized by the grafting respective alcohols to ferulic acid according to the procedure described by Sørensen et al.<sup>14</sup> Ferulic acid (C0) and ethyl ferulate (C2) were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). Standards for quantification of volatile lipid oxidation products were from Sigma-Aldrich (Steinheim, Germany).

**Production of fish oil enriched milk with different antioxidants.** Milk with 1.5% and 0.4% fat content were mixed (6:5, w/w) to obtain a total fat content of 1%. Milk was then heated to 72 °C and kept for 15 sec. The selected temperature and time is known to get a better oxidative stability of the fish oil enriched milk according to a previous study.<sup>15</sup> Fish oil (0.5%, w/w) was then added to the milk while stirring manually. The fish oil enriched milk was homogenized with a two-valve homogenizer (Rannie, Albertslund, Denmark) with a pressure of 225 bar and recirculated for 3

times. To have enough fish oil enriched milk for all sample codes and sampling times, two batches (3 kg each batch) were prepared and then pooled.

The fish oil enriched milk was then divided into 1000 ml autoclaved bottles and the different methanolic antioxidant solutions (C0, C1, C2 and C12) were added to 4 bottles separately to make 100  $\mu$ M final concentration. For the sample codes with combination of two ferulates, C1 (50  $\mu$ M) or C2 (50  $\mu$ M) was combined with C12 (50  $\mu$ M) i.e. total antioxidant concentration 100  $\mu$ M. For the control (Con), only methanol was added in same amount as the antioxidant solutions. After gently shaking, the bulk samples of the different emulsions were then divided into 100 mL sterilized bottles for storage at 5 °C in darkness. On day 0, 3, 6, 9 and 13, one bottle of each code samples were taken out and divided into 50 ml brownbottles, flushed with nitrogen and stored at -40 °C for chemical analysis. On day 1 and day 13, droplet size of each sample code was measured without prior freezing of the samples. On day 1, 100 mL of each treatment except Con was taken out to determine the partitioning of ferulic acid and ferulate esters in the fish oil enriched milk.

## Methods of Analysis

*Droplet size measurement.* A few drops of the sample were suspended in recirculating distilled water (2800 rpm, obscuration 13-15%, method: Fraunhofer) to measure the lipid droplet size in the fish oil enriched milk by a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Triplicate samples were measured.  $D_{3,2}$  was used to report the results.

*Lipid extraction by Bligh and Dyer method.* Lipid from fish oil enriched milk samples was extracted by Bligh and Dyer method.<sup>16</sup> Briefly, milk sample was homogenized with methanol, chloroform and water (1:2:2:1, w/v/v/v). After phase separation by centrifugation, the chloroform layer which



contained lipid was kept for analysis. For each treatment, duplicate lipid extractions were performed and analyzed for peroxide value (PV), fatty acid composition and tocopherols.

*PV analysis.* Lipid extracts from the samples were evaporated to dryness under nitrogen. PV analysis was performed on the same day of lipid extraction based on a method described by Shantha and Decker.<sup>17</sup> Results were reported as meq peroxides/kg lipids.

*Fatty acid composition analysis.* Lipid extracts were evaporated to dryness under nitrogen before analysis. 100  $\mu$ L internal standard C23:0 in heptane, 200  $\mu$ L heptane with BHT and 100  $\mu$ L toluene were added. Lipids were methylated with boron trifluoride reagent (20%) by a one-step procedure using a microwave (Multiwave 3000 SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. Samples were heated for 5 min at 500 W and cooled down for 10 min. Then it was mixed with 1 mL saturated NaCl solution and 0.7 mL heptane. The top layer was used for analysis by gas chromatography with flame ionization detection (GC-FID) (HP 5890A, Agilent Technology, Palo Alto, CA) according to AOCS official Method Ce 1b-89.<sup>18</sup> A DB-wax column (10 m $\times$ ID 0.1 mm  $\times$  0.1  $\mu$ m film thickness, J&W Scientific, Folsom, CA) was used for analysis. The oven temperature program was set initially as 160  $^{\circ}$ C and increased gradually to 200  $^{\circ}$ C at 10.6  $^{\circ}$ C/min, kept at 200  $^{\circ}$ C for 0.3 min, and increased to 220  $^{\circ}$ C at 10.6  $^{\circ}$ C/min, kept at 220  $^{\circ}$ C for 1 min, then increased to 240  $^{\circ}$ C at 10.6  $^{\circ}$ C/min and finally kept at 240  $^{\circ}$ C for 3.8 min. Injection volume is 0.2  $\mu$ L with a split ratio of 1:50. Results were reported as percentages of total fatty acids.

*Tocopherol concentration determination.* Tocopherol concentration in samples were analyzed based on the AOCS official method Ce 8-89.<sup>19</sup> Briefly, lipid extracts were evaporated to dryness under nitrogen and dissolved in 1 mL heptane and directly analyzed by HPLC (Agilent 1100 series, Agilent Technology, Palo Alto, CA) with a fluorescence detector. The different tocopherols were separated on a silica column (Waters, 4.6 mm $\times$ 150 mm, 3  $\mu$ m). The mobile phase was isopropanol

in hexane (0.5:99.5, v/v). The excitation wavelength and the emission wavelength were set at 290 nm and 330 nm respectively. Results were reported as  $\mu\text{g}$  tocopherol/g lipid.

*Volatile compound analysis.* A dynamic headspace gas chromatography mass spectrometry (GC-MS) method was used to analyze the volatile compounds of the milk samples. 4 g of milk samples with 0.5 mL of antifoam (Synperonic, 800  $\mu\text{L/mL}$  water) was put in a pear shaped bottle and heated in a water bath at 45  $^{\circ}\text{C}$  for 30 min. A nitrogen flow of approximately 150 mL/min was used to purge the milk sample in order to release the volatile compounds from the milk into a Tenax GR packed tube. Tenax GR tubes were put in an automatic thermal desorber (ATD-400, Perkin Elmer, Waltham, MA) to desorb the volatiles. Volatile compounds were then transferred automatically from ATD to a GC (Agilent 6890, Palo Alto, CA) with a mass selective detector (HP 5973). A DB1701 column (30 m, 0.25 mm  $\times$  1 mm film thickness, J&W Scientific, Folsom, CA) was used to separate the volatile compounds. The oven temperature was kept at 45  $^{\circ}\text{C}$  for 5 min, increased to 55  $^{\circ}\text{C}$  at 1.5  $^{\circ}\text{C/min}$ , and increased from 55  $^{\circ}\text{C}$  to 90  $^{\circ}\text{C}$  at 2.5  $^{\circ}\text{C/min}$ , then increased to 220  $^{\circ}\text{C}$  at 12  $^{\circ}\text{C/min}$  and finally kept at 220  $^{\circ}\text{C}$  for 4 min. Compounds were identified using the NIST data base and also confirmed with authentic standards. Volatile compounds in the samples were then quantified according to the external standard calibration curves using the ChemStation software. 30 mg of mixed external standard solutions ranged from 0.1  $\mu\text{g/g}$  to 10  $\mu\text{g/g}$  was added to 4 g fresh milk (1.5% fat content) and also analyzed in the same way to prepare the calibration curves. Each sample was analyzed in triplicate and results were reported as ng volatile/g milk.

*Protein carbonyls analysis.* Protein oxidation in the milk samples was evaluated by carbonyl analysis based on the method by Levine et al.<sup>20</sup> and Fenaille et al.<sup>21</sup> Briefly, 50  $\mu\text{L}$  of milk was incubated with 0.5 mL 10 mM DNPH in 2 M HCl for 30 min in dark at room temperature. For the same milk sample a control was performed (0.5 mL 2 M HCl without DNPH). Milk proteins were precipitated with 10% (w/v) TCA (final concentration) and recovered by centrifugation. Protein

pellets were washed 3 times with 1 mL of ethanol/ethyl acetate 50:50 (v/v) to completely remove free DNPH reagent. The pellet was redissolved in 1 mL of 6 M guanidine hydrochloride (pH 2.3). Protein carbonyls and protein content were determined by UV at 370 nm and 280 nm respectively using a spectrophotometer. An extinction coefficient of  $2.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  was used for the calculation of carbonyls. Results were expressed as nmol carbonyl/mg protein (n=3).

*Determination of the partitioning of ferulic acid and ferulates in fish oil enriched milk.* A simple ultracentrifuge procedure based on the method described by Jacobsen et al. with modification<sup>22</sup> was used to determine the partitioning of ferulic acid and ferulate esters in fish oil enriched milk samples. Triplicate samples of 20 mL were ultracentrifuged (Beckman Coulter Inc., Palo Alto, CA) for 2 h at 15 °C at 110,000 g. After centrifugation, samples were frozen at -18 °C to facilitate the separation of the obtained phases. There were 3 phases obtained after centrifugation: oil and emulsion (containing milk fat globule membrane), aqueous, and precipitate. Oil and emulsion phase was removed carefully by a sharp small spatula. Aqueous phase and precipitate could be poured out from the centrifuge tubes directly. Each phase was weighed and diluted with ethanol (1:4) and sonicated for 1 min except aqueous phase. Aqueous phase was directly filtered through a 0.45 µm PVDF syringe filter and analyzed. The mixed solution was then centrifuged and the supernatant was also filtered through the same type filter before HPLC analysis.

Ferulic acid and ferulate esters were determined by HPLC (Agilent 1100 series, Agilent Technology, Palo Alto, CA) at 328 nm based on the method described by Guyot et al.<sup>23</sup> with modification. An ODS-3 column (250\*4.6 mm, 5µm; Phenomenex, Torrance, CA) reversed phase column was used for the determination for all the samples. Elution was obtained by using a mobile phase of solvents A [water (pH 3)] and B [methanol/acetonitrile (1:1)]. Gradient elution was as follows: 95:5 (A/B) for 2 min then to 60:40 (A/B) over 3 min, to 0:100 (A/B) over 10 min and 0:100 (A/B) for 5 min, then reversion back to 95:5 (A/B) over 1 min, finally 95:5 (A/B) for 4 min. The flow rate was 0.9

mL/min. Standard solutions of ferulic acid and ferulates were analyzed under the same HPLC conditions to identify and quantify the ferulic acid and ferulates in each sample prepared from different phases.

**Statistical Analysis.** All data shown represent the mean value  $\pm$  standard deviation of duplicate or triplicate measurements. Data from the different quality parameters were subjected to one-way analysis of variance using SPSS software (version 19.0; SPSS Inc., Chicago, IL). Comparison of means was performed using a least squares difference (LSD) method ( $P < 0.05$ ). For PV or volatile compound results, we use the term “synergistic” effect of C1(C2) with C12 when there was no statistical difference between C1(C2)+C12 and C1(C2), but when the values of the combination were significantly lower than C12. Only when C1(C2)+C12 showed statistical significance when compared with C1(C2) and C12, they were compared with the expected values to determine the “synergistic” effects. For PV and volatile compound analysis results, expected C1(C2)+C12 were calculated by the average of C1(C2) with C12. This approach to evaluate synergistic effect is somewhat different from the commonly used approach where a synergistic effect is considered when the sum of the effects of individual compounds each added in a concentration of x and y is lower than the effect of the two compounds added together in a concentration of x+y. The effect is usually measured as % inhibition of the measured parameters. To highlight that a different approach has been used in this study the term synergistic is quoted.

## RESULTS AND DISCUSSION

**Droplet size of the lipid in milk emulsion system.** Overall, the droplet size of the lipid in all the samples increased from an average of 0.81  $\mu\text{m}$  to 0.87  $\mu\text{m}$  during the whole storage period (data not shown). No creaming or flocculation was observed during 13 days of storage at 5 °C. The lipid droplet size of the fish oil enriched emulsion was slightly higher than in our previous studies.<sup>12,13</sup> It

may be related with the natural variation in the composition of milk used in the different studies. Particle size can range from 0.5 to 1.6  $\mu\text{m}$  in fish oil enriched milk samples depending on the homogenization temperature and pressure.<sup>24</sup> Particle size can influence the oxidation stability of emulsion as reported by Let et al.<sup>15</sup> This is not the focus of the present research and has been investigated before. Moreover, differences in droplet sizes were not significant and most likely did not affect lipid oxidation.

**Fatty acid composition.** EPA contents of all the samples on day 0 were from 3.54 - 3.70% and 3.41 - 3.74% on day 13. DHA contents changed from 4.26 – 4.72% on day 0 to 4.13 – 4.45% on day 13 (data not shown). No significant reduction of EPA and DHA content was observed for samples between day 0 and day 13 except slight reduction for the C0 sample. EPA and DHA are prone to oxidation due to their highly unsaturated nature. In previous studies, the reduction could be significant or non-significant depending on the extent of lipid oxidation.<sup>11,12</sup>

**Change of PV in fish oil enriched milk emulsions.** In general, PV of the control sample increased dramatically from day 0 to day 6 and then maintained stable values (Figure 1). For both C0 and C12 samples, PV increased gradually from day 0 to day 9 at a rate of 0.98 and 0.77 meq peroxides / kg lipid per day, respectively and then became steady. Control sample had significantly higher values than both C0 and C12 sample from day 3 till the end of the storage, while C0 had significantly higher PV than C12 after day 6 ( $P<0.05$ ). C1+C12 and C2+C12 had very similar slightly increasing PVs during the whole storage period, in agreement with the very similar PVs of C1 and C2. From day 6, the combination of C1 or C2 with C12 had significantly higher PVs than either C1 or C2, but lower PVs than C12 and the expected combination values indicated as dashed lines in Figure 1 ( $P<0.05$ ). The lower PVs than expected values based on the individual treatments indicated a “synergistic” effect of the combination of ferulates with short and medium chain lengths. Further studies are required to confirm this preliminary finding on the “synergistic” effect and

synergistic effect with the use of other chain lengths or type of phenolipids. Furthermore, the synergistic effect should be investigated at other concentrations than that applied in the present study. C1 and C2 showed a very slight increase in PV throughout the storage period and had the lowest PV compared to all other treatments. However, the low PV did not necessarily indicate low lipid oxidation as the peroxides may be transformed into secondary oxidation products. The secondary oxidation products results presented next could provide a better profile of the lipid oxidation in all the treatments.

**Change of volatile secondary oxidation products during storage.** Secondary oxidation products are mainly derived from the decomposition of primary lipid hydroperoxides. Hexanal, 1-penten-3-one, 1-penten-3-ol, 2,4-heptadienal, 2-pentenal, 1-hexanol, 2,6-nonadienal were quantified. Figure 2 shows the changes of hexanal, 1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal. They represent the general development trend of the volatile secondary compounds quantified during storage. In addition, 1-penten-3-one, 1-penten-3-ol, 2,4-heptadienal were found to be related to the off flavor generated by oxidation of n-3 PUFAs.<sup>25</sup>

Hexanal are mainly from the oxidation of n-6 fatty acids and is a very important index of lipid oxidation. Both fish oil and milk fat contain significant amount of n-6 fatty acids, which are easily oxidized due to the unsaturated nature. Hexanal in all the samples reached the peak on day 6 and then gradually decreased or remained constant in concentration (Figure 2). Corresponding to the PV results, all the treatment had much lower concentrations than the control sample while C0 and C12 had much higher concentrations than all other ferulate treatments after day 3 ( $P<0.05$ ). These results confirmed the strongest antioxidant effects of lipophilized ferulic acid with short alkyl chain lengths. In addition, there was no significant difference between C12 and C0 with regard to PV until day 9, however, C12 sample had significantly lower hexanal content than C0 sample starting from day 6 ( $P<0.05$ ). This finding suggested that C12 and other shorter lipophilized ferulic acid could

have stronger antioxidant effects by inhibiting not only primary oxidation but also the secondary oxidation reactions compared to ferulic acid. This is particularly important as the volatile compounds due to oxidation could cause off flavor of the food products. On day 13, the ranking order of the concentration of hexanal was:  $\text{Con}^a > \text{C0}^b > \text{C12}^c > \text{C1} + \text{C12}^d = \text{C2} + \text{C12}^d = \text{C1}^d = \text{C2}^d$ . The hexanal concentration in  $\text{C1} + \text{C12}$  or  $\text{C2} + \text{C12}$  was not significantly higher than C1 or C2, but significantly lower than C12, suggesting a “synergistic” effect of the combination of C1 or C2 with C12 on the reduction of hexanal content.

The content of 1-penten-3-one in all the samples also reached a peak concentration on day 6 except for C2 sample (Figure 3). In contrast to hexanal, 1-penten-3-one decreased sharply after day 6 and reached to approximately the same level for all the treatments at the end of the storage. The decline of 1-penten-3-one in fish oil enriched milk samples at later stage of storage period was also found in other similar studies.<sup>4,11,13</sup> C0 and C12 did not show lower concentration of 1-penten-3-one compared to the control until day 6 while the other treatments had significantly lower 1-penten-3-one concentration compared to the control starting from day 3 ( $P < 0.05$ ). The biggest difference in 1-penten-3-one concentrations among all treatments was shown on day 6. C2 had the lowest 1-penten-3-one concentration among all the treatments ( $P < 0.05$ ). C1,  $\text{C1} + \text{C12}$  and  $\text{C2} + \text{C12}$  showed similar antioxidant effects ( $P > 0.05$ ). The dramatic decline of 1-penten-3-one at the end of storage period was probably related to its reaction with other components in the milk sample.<sup>11</sup>

1-penten-3-ol is developed during the decomposition of hydroperoxides of the n-3 polyunsaturated fatty acids.<sup>26</sup> In contrast to 1-penten-3-one, 1-penten-3-ol increased steadily during the whole storage period after a lag phase of 3 days (Figure 4). Starting from day 6, the concentration of 1-penten-3-ol in both C2 and C1 had more than 50% reduction compared to the control. On day 13, the ranking order of the sample codes based on the concentration of 1-penten-3-ol was:  $\text{Con}^a > \text{C0}^b = \text{C12}^b > \text{C1} + \text{C12}^c = \text{C2} + \text{C12}^c > \text{C1}^d = \text{C2}^d$ . Moreover, the concentration of 1-penten-3-ol in both

270 C1+C12 and C2+C12 were lower than their expected values, respectively ( $P<0.05$ ) (data not  
271 shown), indicating a “synergistic” effect of the combination of C1 or C2 with C12.

272 Corresponding to the increase in PV, a dramatic increase in 2,4-heptadienal for Con and C0 samples  
273 were observed after day 3, while a much slower increase was observed in all the ferulate treatments  
274 (Figure 5). At the end of storage, the ranking order of the sample codes based on the concentration  
275 of 2,4-heptadienal was:  $\text{Con}^a = \text{C0}^a > \text{C12}^b \geq \text{C1+C12}^{bc} = \text{C2+C12}^{bc} \geq \text{C1}^c = \text{C2}^c$ . A “synergistic” effect  
276 could not be determined due to the relatively high standard deviations.

277 Overall, for all the compounds quantified in the present study, C2 and C1 had the lowest  
278 concentration for all the volatile compounds followed by C2+C12, C1+C12, C12 and C0. Con had  
279 the highest concentration of the analyzed volatile compounds. This kind of difference was also  
280 observed in the PV results. These findings clearly demonstrated that lipophilization of ferulic acid  
281 with short alkyl chain lengths caused a dramatic increase of the antioxidant activity, but the  
282 antioxidant activity was sharply reduced with medium alkyl chain lengths in fish oil enriched milk.  
283 In addition, an indication of a “synergistic” antioxidant activity of ferulates with short and medium  
284 alkyl chain lengths were observed as shown in PV, hexanal and 1-penten-3-ol results.

285 **Change of tocopherol in the fish oil enriched milk samples.** Tocopherol of the milk samples  
286 mainly originated from the fish oil used in the study. The samples can be separated into three  
287 groups roughly based on the changing trend of  $\alpha$ -tocopherol contents (Figure 6). During the whole  
288 storage period, C1 and C2 treatments had the lowest  $\alpha$ -tocopherol reduction while  $\alpha$ -tocopherol  
289 levels in C1+C12 and C2+C12 were reduced moderately. C0, C12 and Con treatments had the  
290 highest reduction. There were significant differences in the content of  $\alpha$ -tocopherol among these 3  
291 groups starting from day 6 except C0 sample on day 6 ( $P<0.05$ ). In general, it showed that changes  
292 in  $\alpha$ -tocopherol levels were well correlated with the change in PV in the samples according to these



3 groups although there was some discrepancy within the highest reduction group (Con, C0, and C12). These findings were also in agreement with previous studies by Alemán et al.<sup>11</sup> and Sørensen et al.<sup>12</sup> There was not as high amount of  $\gamma$ -,  $\delta$ -tocopherol as  $\alpha$ -tocopherol in the samples. The reduction trend of  $\gamma$ -tocopherol was not as significant as for  $\alpha$ -tocopherol, and there was no obvious trend in the change of  $\delta$ -tocopherol (data not shown).

**Protein oxidation in fish oil enriched milk.** Changes in protein carbonyls in various milk samples are shown in Figure 7. Increase in carbonyls indicates protein oxidation during storage.<sup>27</sup> The amount of protein carbonyls in the control samples increased significantly during the storage from day 0 to day 13 ( $P<0.05$ ), corresponding to the dramatic increase of PV (Figure 1). The combination of C1 or C2 with C12 also showed inhibition effects for protein oxidation throughout the storage. Although C12 showed stronger lipid oxidation inhibition effects than C0, this was not the case for protein oxidation. C2 had the lowest carbonyls among all the treatments at the end of the storage ( $P<0.05$ ).

The protein oxidation observed by increase of carbonyls could be caused by the lipid oxidation products as these two reactions often occur simultaneously. Reactive oxygen species generated from lipid oxidation process can react with protein and cause protein oxidation.<sup>28</sup> C1 and C2 showed strong inhibition of lipid oxidation and consequently protein oxidation. The contrast in lipid and protein oxidation results observed with C12 and C0 suggested that C0 could exhibit extra protection of protein from oxidation other than by reducing the lipid oxidation products. This could be related to the difference of hydrophobicity of C12 and C0 and thus their different partitioning in the milk sample as presented below. Further research such as loss of sulphydryl content is needed to confirm the effects of lipophilized ferulate esters on protein oxidation. In addition, research on whether 1-penten-3-one generated in the lipid oxidation process could react with protein could help to understand the mechanism of protein oxidation.

**Partitioning of lipophilized ferulates in the fish oil enriched milk.** Milk samples were physically separated into 3 different phases after ultracentrifugation: oil and emulsion, aqueous, and precipitate. The oil and emulsion phase was the top layer after ultracentrifugation and consisted of small amounts of oil and milk fat globule membrane. It was difficult to completely separate the oil from emulsion by the method used in this study, and this is the reason for the combination of them into one phase. The precipitate phase contained proteins both from the aqueous phase and the O/W interface layer. Approximately 61.9% of C12 partitioned in the oil and emulsion phase, followed by C2 (50.8%) and C1 (40.3%), while C0 (13.2%) had the lowest percentage in this phase ( $P<0.05$ ) (Figure 8). This is in agreement with their corresponding hydrophobicity. In the aqueous phase, C0 had the highest percentage (70%). Unexpectedly, C12 which is much more hydrophobic than either C1 or C2 had higher percentage in the aqueous phase than C1 or C2 ( $P<0.05$ ). However, this could be explained as some of C12 could have formed aggregates in the aqueous phase.<sup>29</sup> C1 and C2 with highest antioxidant effects had the lowest partitioning percentages in the aqueous phase. Some studies also found that antioxidants with high efficacies have low partitioning coefficients in the aqueous phase in simple O/W emulsions.<sup>30</sup> This study showed that this phenomenon could also happen in complex food systems such as fish oil enriched milk. Contrary to the aqueous phase, C1 and C2 had relatively higher concentration in the precipitate phase than C12 and C0 ( $P<0.05$ ). Based on their antioxidant effects of these compounds, we may hypothesize that the higher partitioning of an antioxidant compound in the precipitate is related to a better antioxidant activity in fish oil enriched milk. Further research on other antioxidant phenolics to test this hypothesis is required. Protein plays an important role in milk as an emulsifier to stabilize the milk fat. The interaction of antioxidant with proteins and particularly those localized in the oil water interface could inhibit the lipid oxidation significantly, which is in agreement with the interfacial concept.<sup>31</sup> The combination of C1 or C2 with C12 seemed to change their relative partitioning behaviors. C12

decreased in the oil phase in both combination samples but increased in either the aqueous phase (C2+C12) or the precipitate phase (C1+C12) ( $P<0.05$ ). Both C1 and C2 had a slight decrease in the oil phase and a slightly higher increase in the protein phase in all the combination samples, which could increase the antioxidant effects of the combination and explain the “synergistic” effects observed by the antioxidant study. However, no statistical significances were found. An improved *in vivo* analysis method to determine the partitioning behavior among the oil, aqueous and the interface phases is required to provide a more clear picture.

**Localization of ferulic acid and lipophilized ferulates in fish oil enriched milk and the antioxidant activity mechanism.** Apart from the chemical properties of one particular antioxidant, its physical location in an emulsion is also important for its effectiveness.<sup>32</sup> C0 was the least effective with regard to lipid oxidation among all the antioxidants tested in this study. As it was reported that milk oil droplets were negatively charged,<sup>33</sup> this could be caused by the negative charge status of this compound in the milk. Consequently, C0 could be electrostatically repelled and have relatively lower concentration at the oil droplet surface than uncharged ferulates. In another study, researchers also showed that phenolic compounds with different electrical charge had an impact on their partitioning and the antioxidant activity in salmon oil-in-water emulsions.<sup>34</sup>

The difference of effectiveness among antioxidants is also probably due to the different hydrophobicity they have. The hydrophobicity of C12 is much higher than C1 or C2. Antioxidant with high hydrophobicity normally showed better antioxidant activity in O/W emulsions.<sup>7,31</sup> However, antioxidant activity of C1 or C2 was significantly higher than C12 from our observation which could be explained by the cut-off effect.<sup>9</sup> The critical chain length of a particular esterified phenolic acid differed in different food system.<sup>11</sup> There were currently three putative hypotheses to explain this cut-off phenomena: the “reduced mobility”, the “internalization”, and the “self-aggregation”, which was described in detail by Laguerre et al.<sup>9</sup> Figure 9 explains the assumed

365 localization of the ferulic acid and lipophilized ferulic acids in fish oil enriched milk based on the  
366 theory that lipid oxidation is initiated at the interface, measured lipid oxidation in the different  
367 sample codes and the partitioning study and how the combination of antioxidants could influence  
368 their localization. From this study, the “internalization” seemed to be the right mechanism to  
369 explain our results. C0 had the highest concentration in the aqueous phase which was showed in  
370 Figure 8 and illustrated in Figure 9A. C2 or C1 had the highest concentration in the precipitate and  
371 could have a relatively higher concentration in the interface due to their lower hydrophobicity  
372 compared to C12 (Figure 9B). A significant amount of C12 could be internalized inside the oil  
373 droplets due to its high hydrophobicity, which was indirectly confirmed by the high percentage of  
374 C12 detected in the oil and emulsion fraction (Figure 9C). Partitioning studies in simplified O/W  
375 emulsion system with rutin laurate and rutin palmitate showed that very low concentrations were  
376 detected in the aqueous phase of the different two or multiple phases systems (buffer / oil phases,  
377 buffer / emulsifier phases and emulsion).<sup>35</sup> This could also support our assumption for the location  
378 of C12 in this study. There is an equilibrium distribution of one particular antioxidant among the oil,  
379 interfacial and aqueous phases. It is assumed that the concentration of each compound in the  
380 interface decides the antioxidant activity because the oil droplet interface is where the lipid  
381 oxidation occurs.<sup>36</sup> The combination of C12 with C1 or C2 could disturb the equilibrium  
382 distribution of C1 or C2 between the oil and interface as also observed in other studies between  
383 tocopherol and caffeates in model emulsions.<sup>37</sup> The combination of C1 or C2 with C12 could lead  
384 to a relatively higher concentration of C1 or C2 in the interface than when C1 or C2 was added in  
385 the fish oil enriched milk emulsion alone (Figure 9D). The higher concentration of C1 or C2 at the  
386 interface can lead to better antioxidant effects, which could explain the indication of the  
387 “synergistic” antioxidant effect observed in this study. In another study, Panya et al.<sup>36</sup> showed that  
388 the equilibrium distribution of antioxidant compound in the oil, interface and aqueous phase could

be broken by adding a surfactant Tween-20 and the antioxidant activity was changed accordingly in O/W emulsions. The present study demonstrated that antioxidant-antioxidant interaction could influence their antioxidant activity in fish oil enriched milk, which could also be related to the change of partitioning coefficients of the antioxidants at the interface. The mechanism of this kind of interaction is still not clear. Challenges include the quantification of distribution of antioxidant compounds in the oil, interfacial, and aqueous phases *in vivo* in a complicated food system like fish oil enriched milk emulsion. In addition, there are also various compounds in a food system that could influence the partitioning of antioxidant in the interface. More research is needed to study the particular factors that could influence the localization of antioxidants and further clarify the “internalization” or “localization” mechanism.

Although partitioning of an antioxidant in the interface of an emulsion system is relevant for its antioxidant activity in inhibiting lipid oxidation, this could not apply to the protein oxidation. Lipid oxidation could originate from the interface and generate primary and secondary lipid oxidation products that react with protein and cause the induction of protein oxidation. An antioxidant such as C1 or C2 that localizes at the interface may inhibit lipid oxidation efficiently and thus protein oxidation. However, antioxidant like C0 which has high concentration in aqueous phase and consequently less efficient lipid oxidation activity could still inhibit protein oxidation. Further research on how C0 could retard the reaction between lipid oxidation products and proteins would help to explain the mechanism.

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#### **Notes**

412 The authors declare no competing financial interest.

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**Figure captions:**

Figure 1. Changes of PV in fish oil enriched milk samples with ferulic acid and different lipophilized ferulates during 13 days storage at 5 °C. Data points and error bars represent means  $\pm$  standard deviations (n=2). Con, control; C0, ferulic acid; C1, methyl ferulate; C2, ethyl ferulate; C12, dodecyl ferulate.

Figure 2. Change of hexanal in fish oil enriched milk samples with ferulic acid and different lipophilized ferulates during 13 days storage at 5 °C. Data points and error bars represent means  $\pm$  standard deviations (n=3). Con, control; C0, ferulic acid; C1, methyl ferulate; C2, ethyl ferulate; C12, dodecyl ferulate.

Figure 3. Change of 1-penten-3-one in fish oil enriched milk samples with ferulic acid and different lipophilized ferulates during 13 days storage at 5 °C. Data points and error bars represent means  $\pm$  standard deviations (n=3). Con, control; C0, ferulic acid; C1, methyl ferulate; C2, ethyl ferulate; C12, dodecyl ferulate.

Figure 4. Change of 1-penten-3-ol in fish oil enriched milk samples with ferulic acid and different lipophilized ferulates during 13 days storage at 5 °C. Data points and error bars represent means  $\pm$  standard deviations (n=3). Con, control; C0, ferulic acid; C1, methyl ferulate; C2, ethyl ferulate; C12, dodecyl ferulate.

Figure 5. Change of 2,4-heptadienal in fish oil enriched milk samples with ferulic acid and different lipophilized ferulates during 13 days storage at 5 °C. Data points and error bars represent means  $\pm$  standard deviations (n=3). Con, control; C0, ferulic acid; C1, methyl ferulate; C2, ethyl ferulate; C12, dodecyl ferulate.

542 Figure 6. Changes of  $\alpha$ -tocopherol in fish oil enriched milk samples with ferulic acid and different  
543 lipophilized ferulates during 13 days storage at 5 °C. Data points and error bars represent means  
544  $\pm$  standard deviations (n=2). Con, control; C0, ferulic acid; C1, methyl ferulate; C2, ethyl ferulate;  
545 C12, dodecyl ferulate.

546 Figure 7. Changes of carbonyl content in fish oil enriched milk samples with ferulic acid and  
547 different lipophilized ferulates during 13 days storage at 5 °C. Data points and error bars represent  
548 means  $\pm$  standard deviations (n=3). Con, control; C0, ferulic acid; C1, methyl ferulate; C2, ethyl  
549 ferulate; C12, dodecyl ferulate.

550 Figure 8. Partitioning of ferulic acid and lipophilized ferulates into 3 different phases in the milk  
551 samples: Oil and emulsion, aqueous and precipitate. Data points and error bars represent means  $\pm$   
552 standard deviations (n=3). C0, ferulic acid; C1, methyl ferulate; C2, ethyl ferulate; C12, dodecyl  
553 ferulate.

554 Figure 9. A schematic illustration of assumed localization and interaction of ferulic acid and  
555 different lipophilized ferulates in fish oil enriched milk: (A) ferulic acid is mainly localized at the  
556 aqueous phase; (B) ferulates with short alkyl chain lengths such as methyl ferulate and ethyl  
557 ferulate have high concentration at the interface; (C) Ferulates with medium alkyl chain lengths are  
558 highly hydrophobic and are mainly in the oil; (D) Interaction between ferulates with short and  
559 medium alkyl chain lengths can change the equilibrium distribution of ferulates with short alkyl  
560 chain length and increase its concentration at the interface.

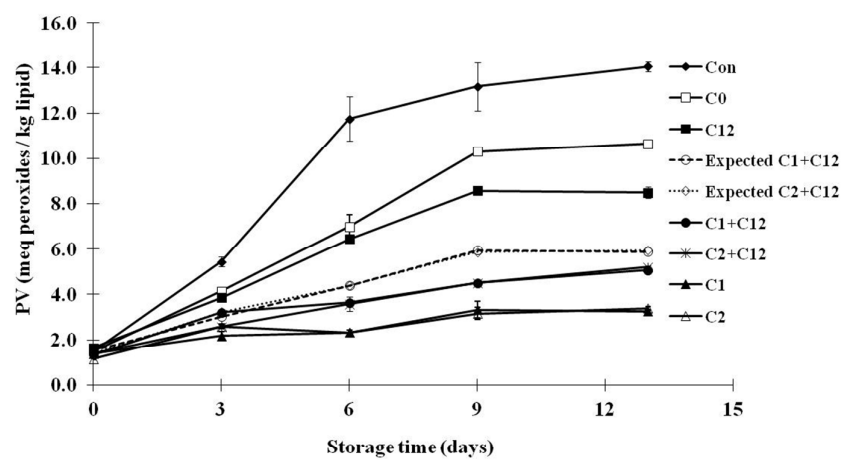


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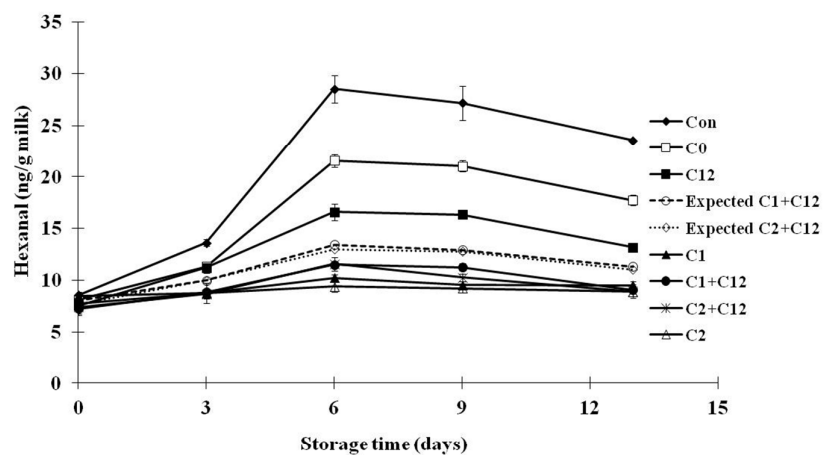


Figure 2

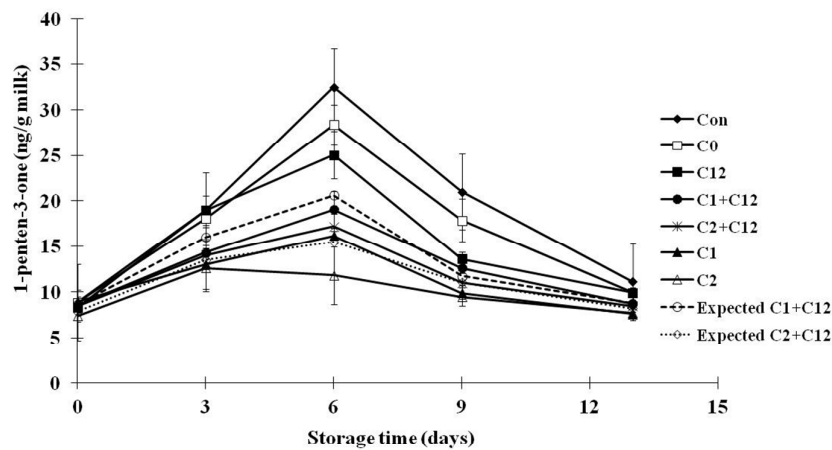


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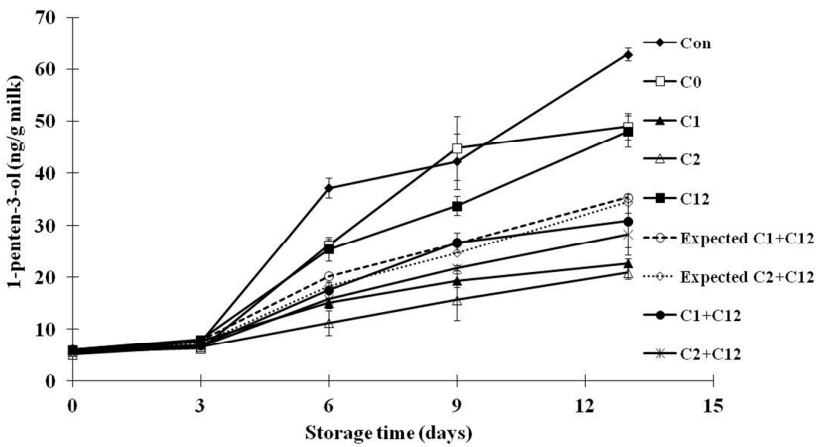


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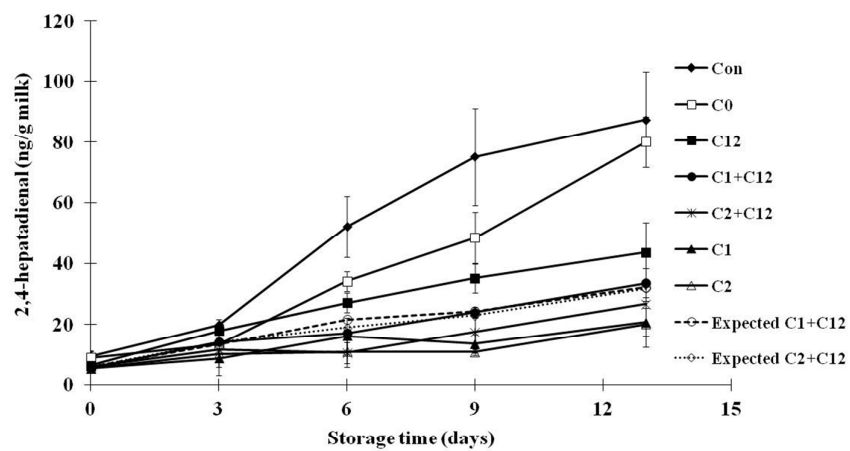


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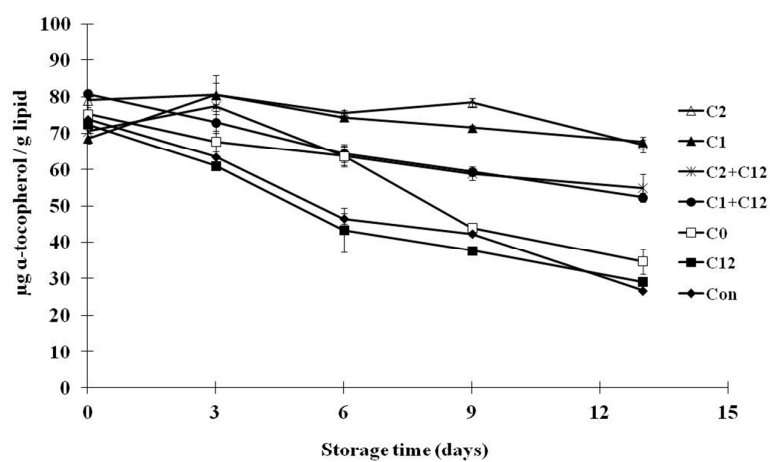


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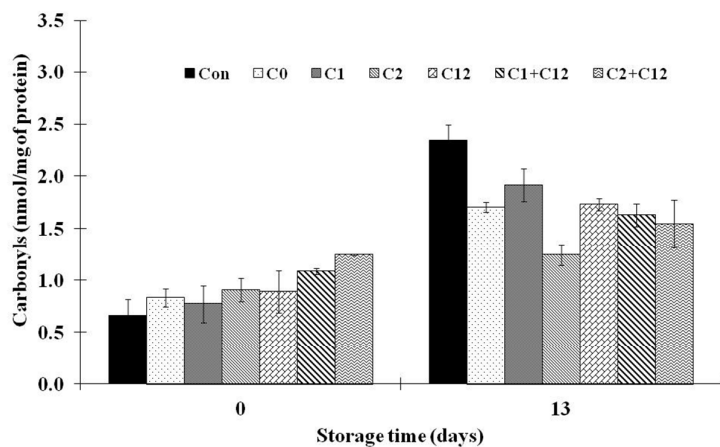


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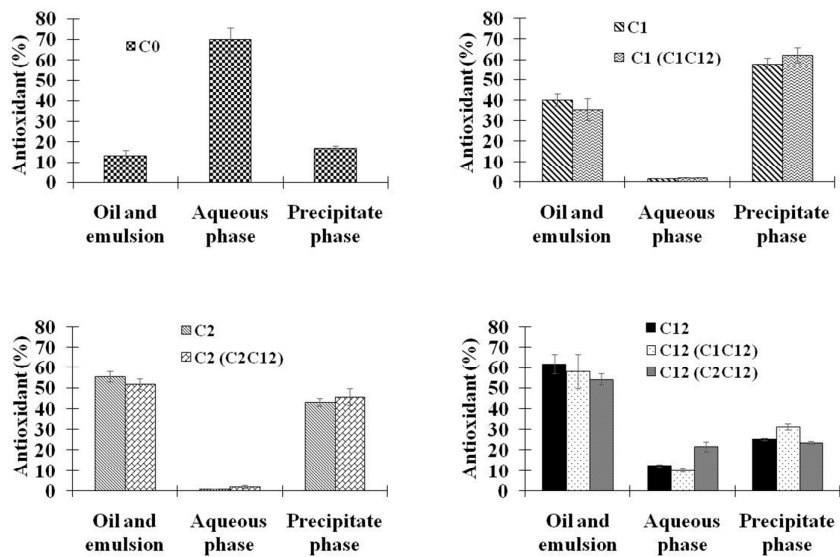


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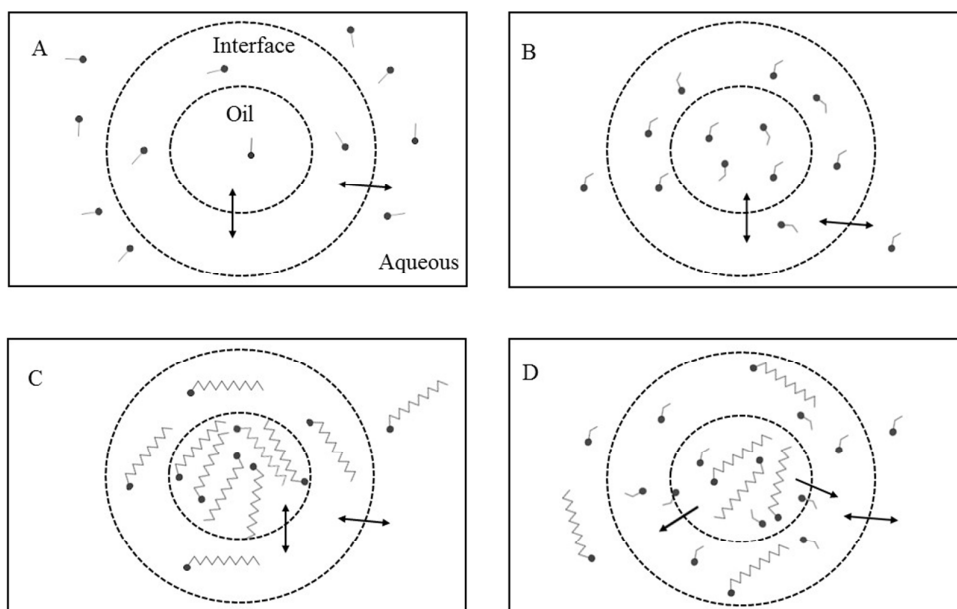
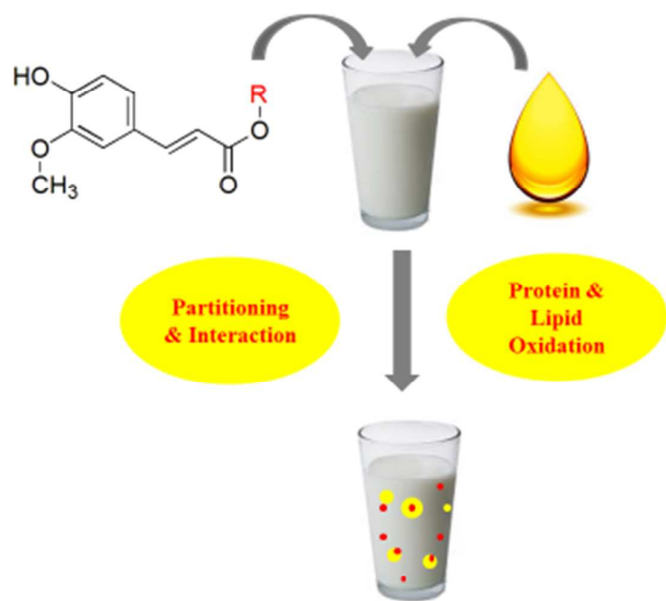
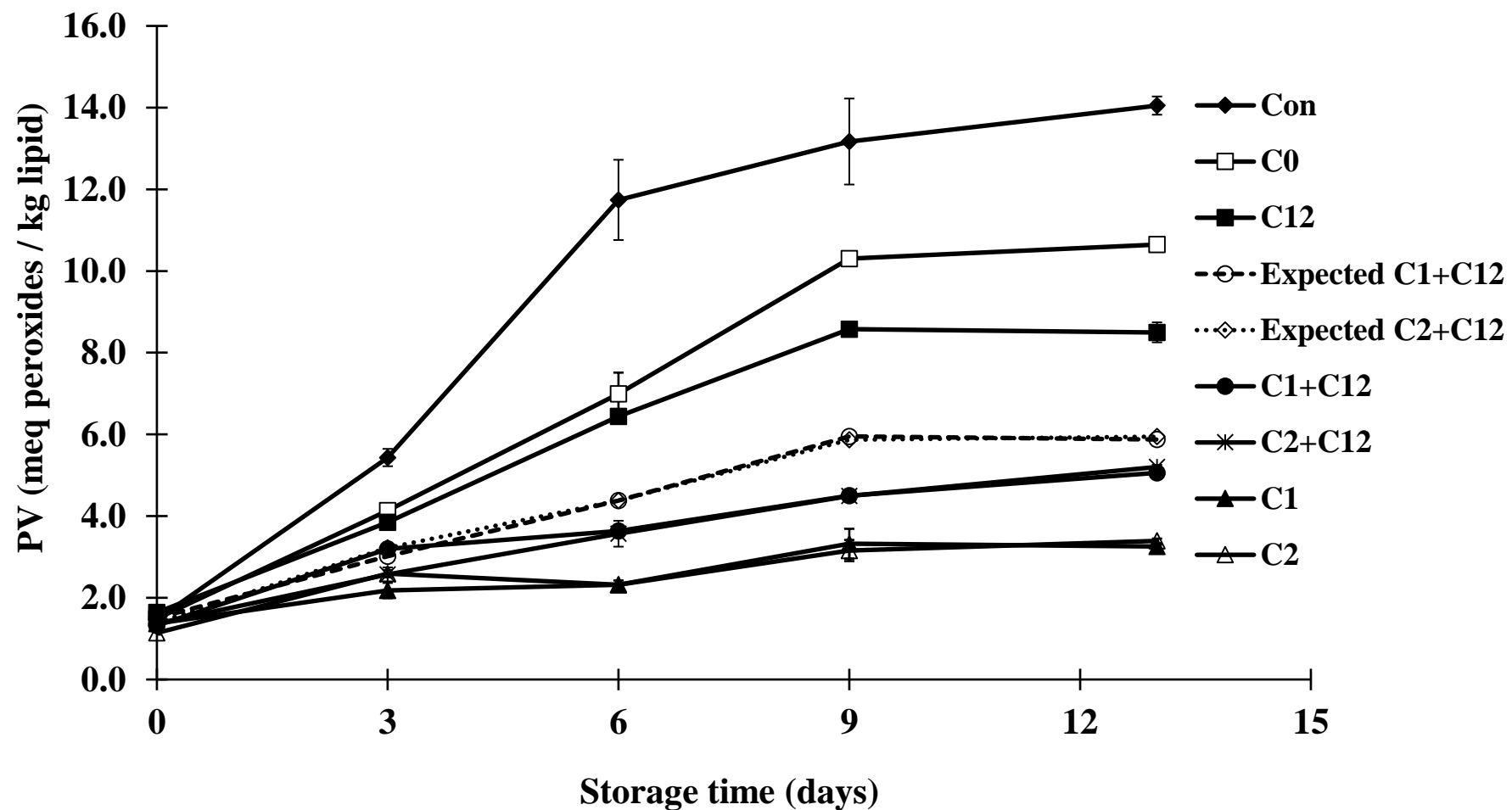
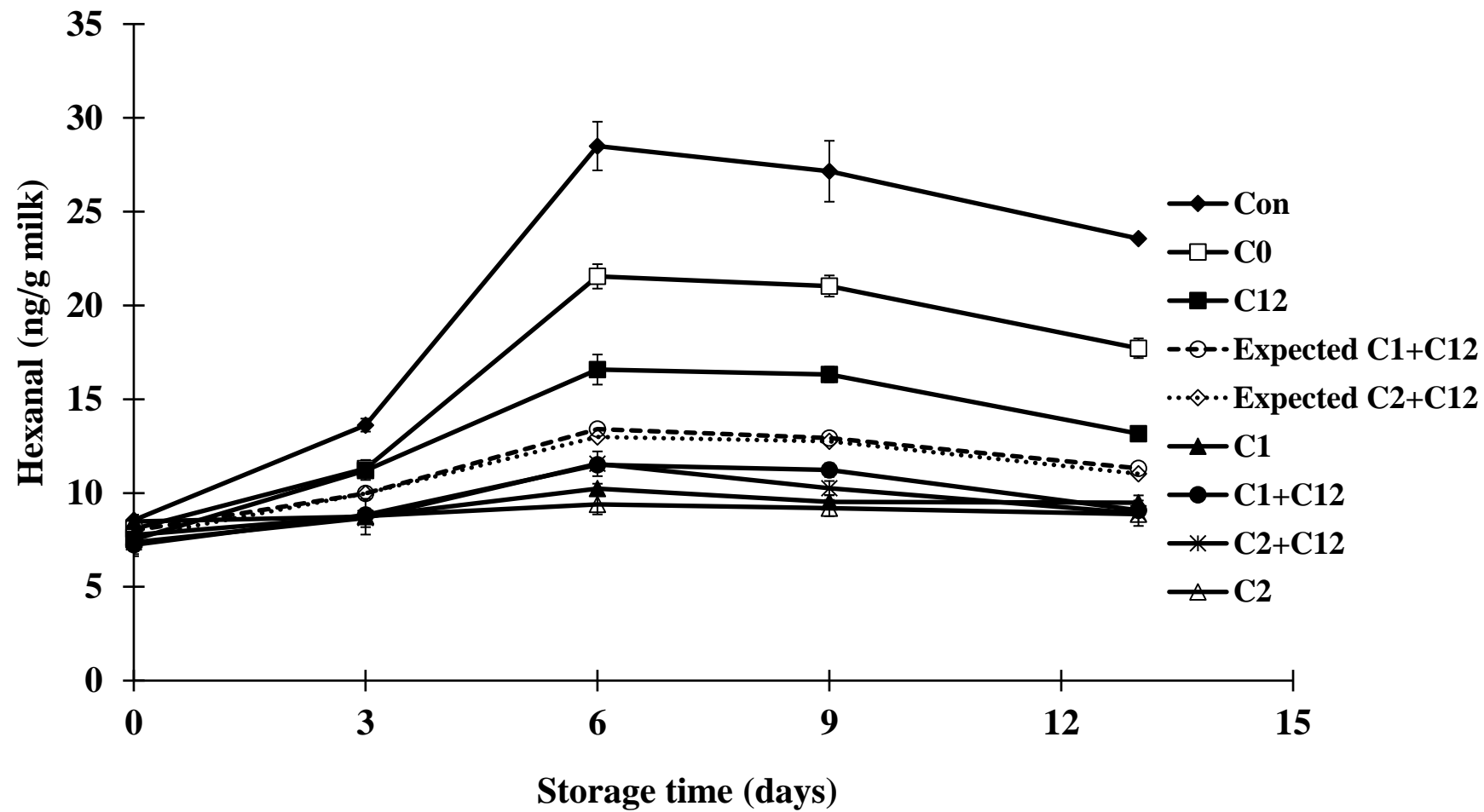


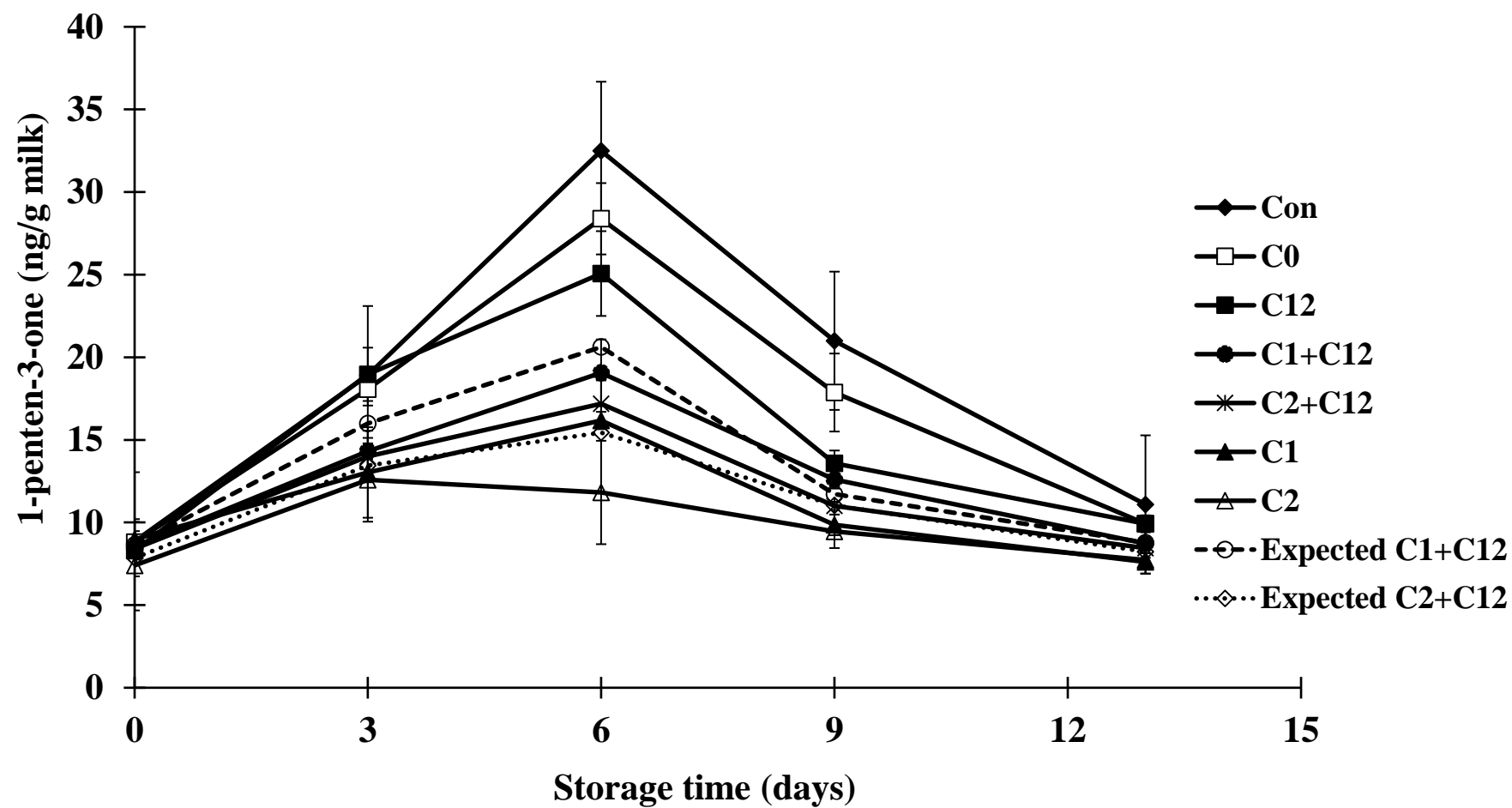
Figure 9

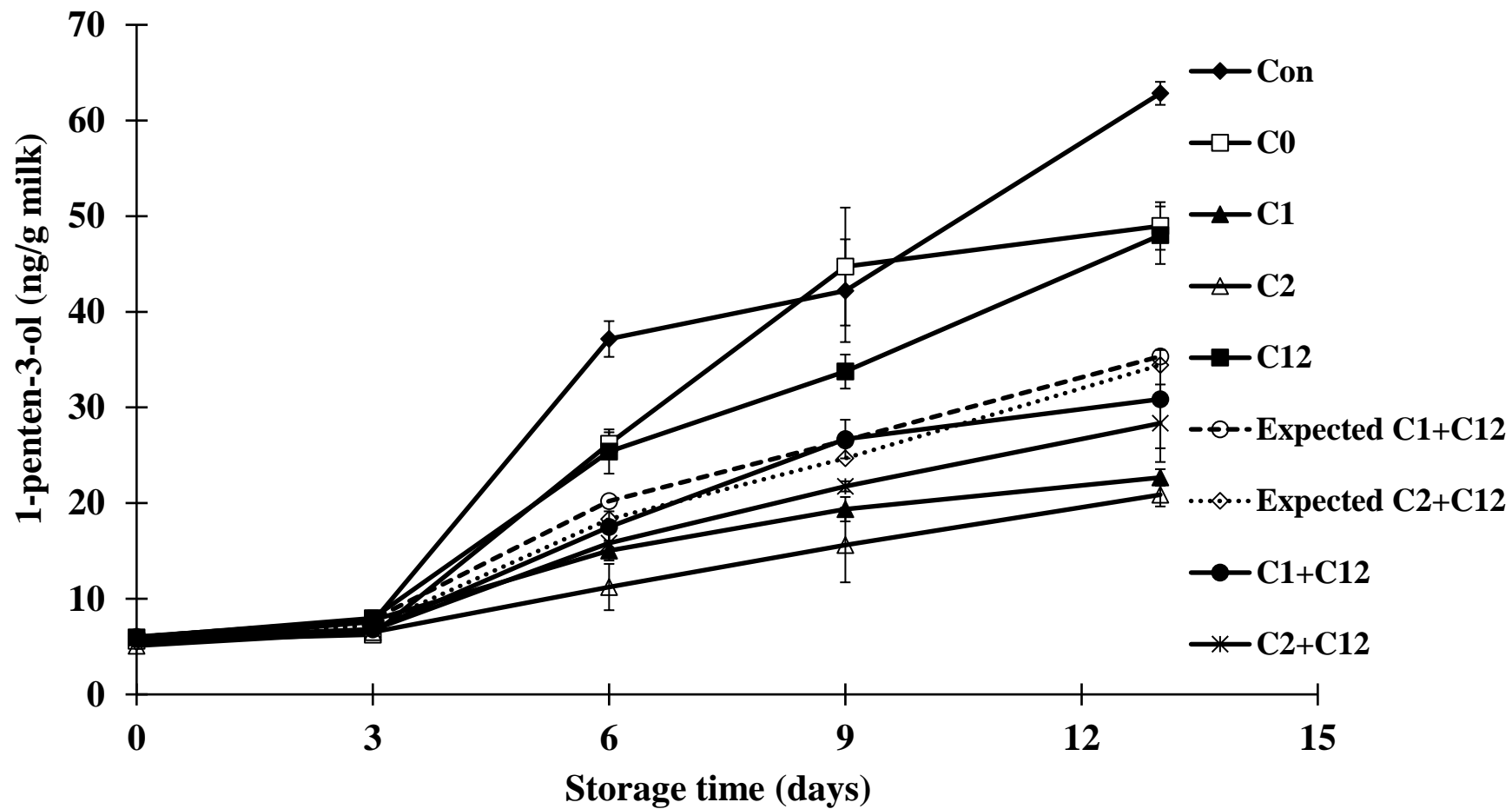
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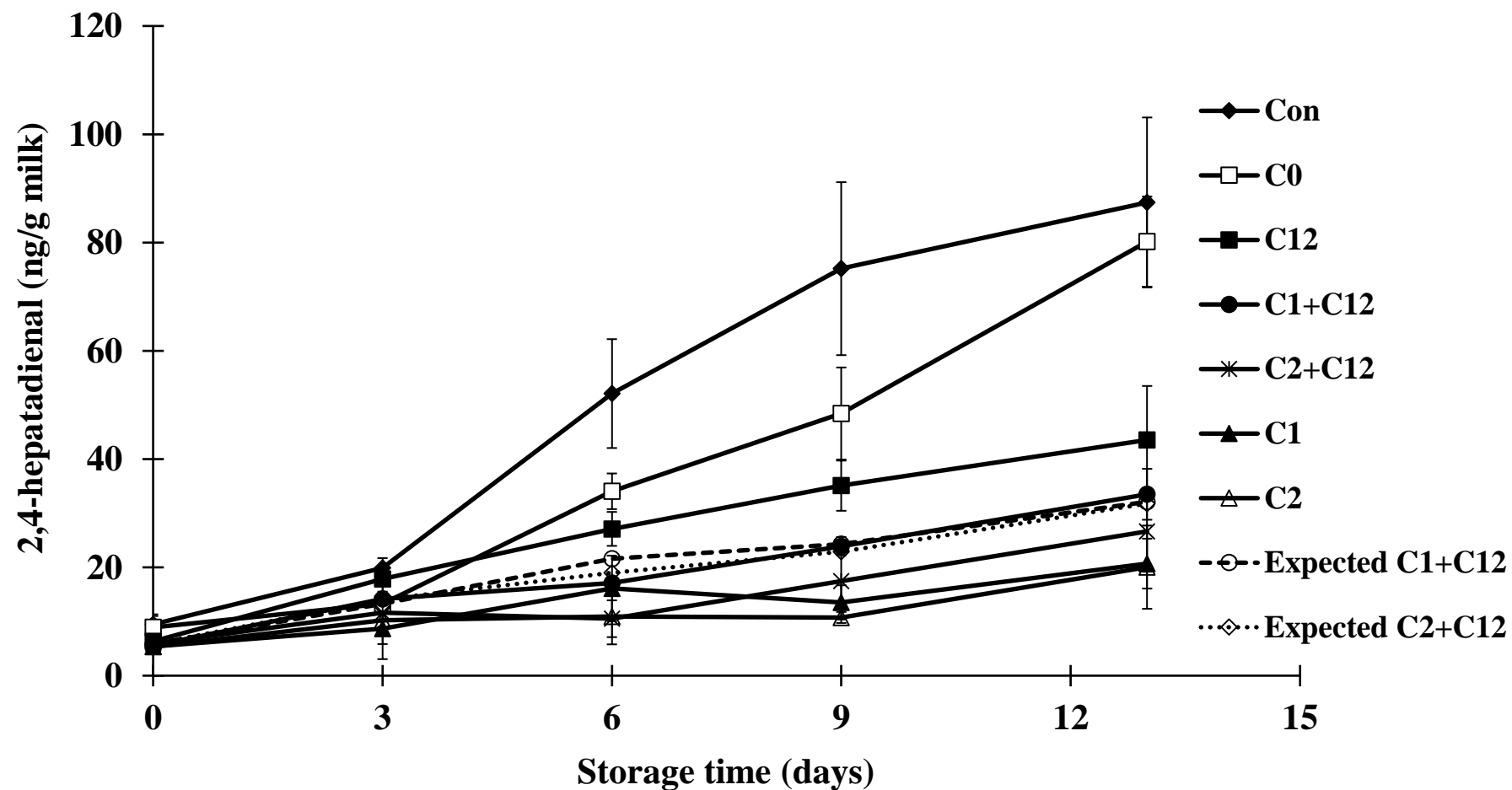




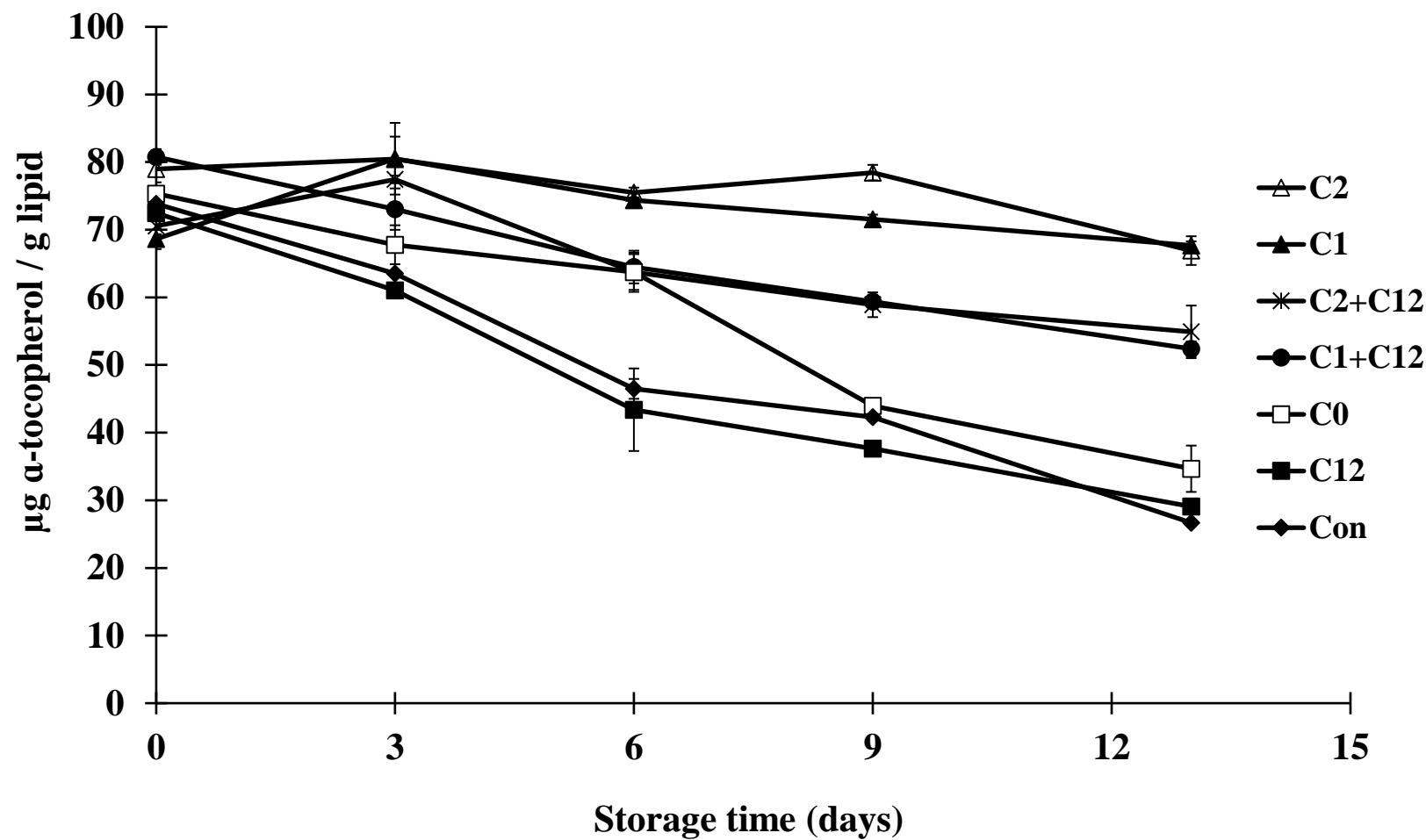


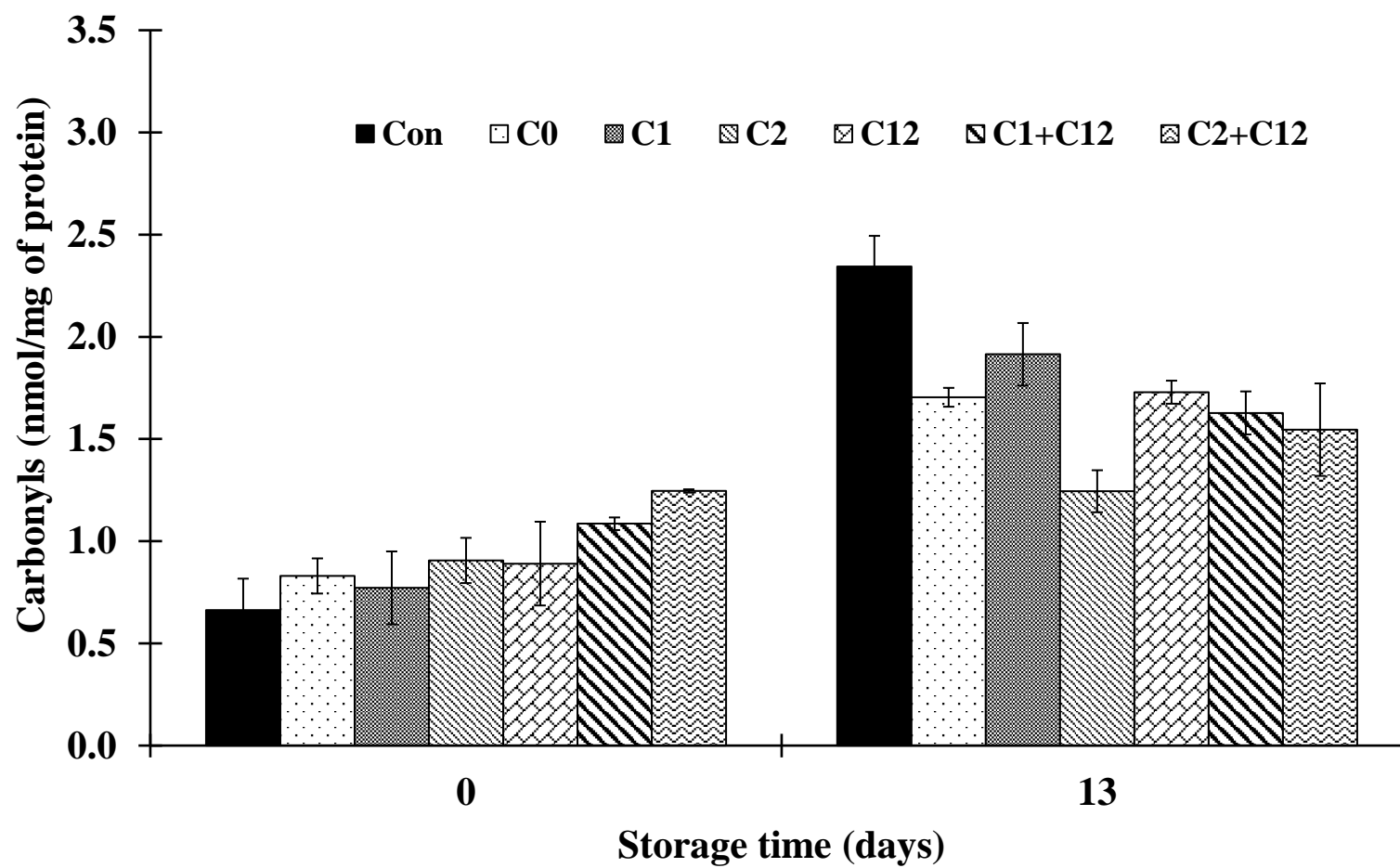


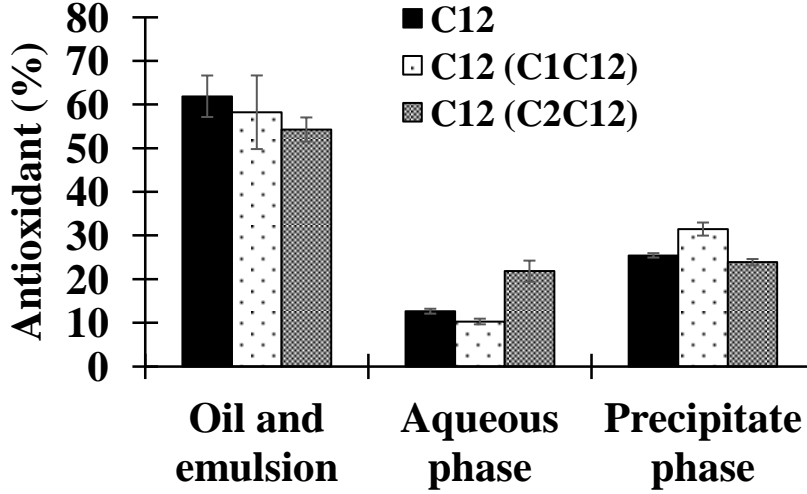
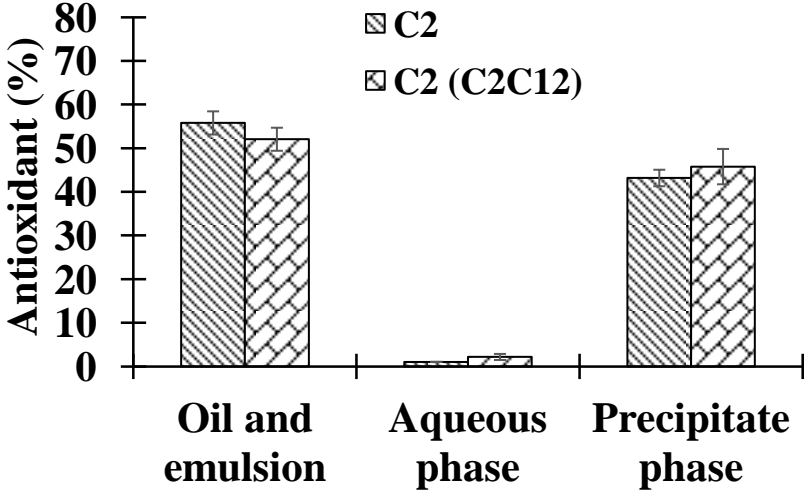
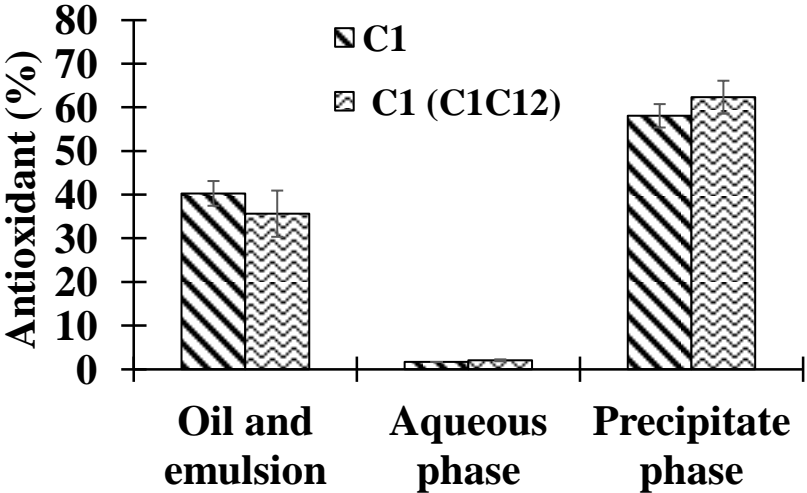
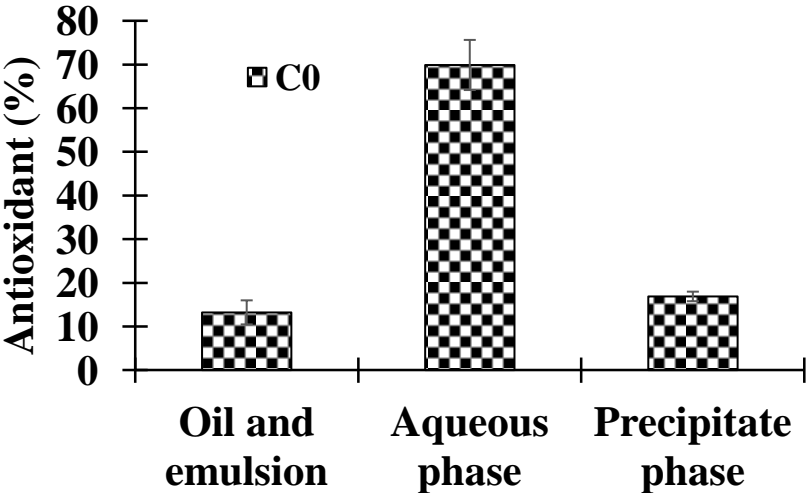


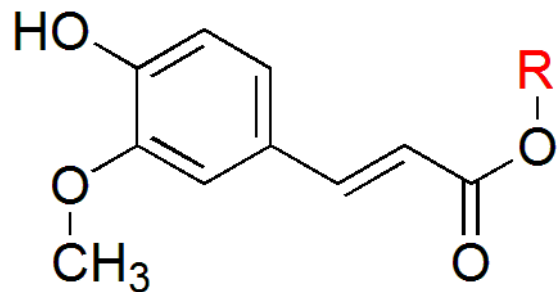












**Partitioning  
& Interaction**

**Protein &  
Lipid  
Oxidation**

